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ORIGINAL PAPER

Immunogenicity of a novel, bivalent, plant-based oral vaccine against hepatitis B and human immunodeficiency viruses

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Abstract A synthetic chimeric gene, *TBI-HBS*, encoding the immunogenic ENV and GAG epitopes of human immunodeficiency virus (HIV-1) and the surface protein antigen (HBsAg) of hepatitis B virus (HBV), was expressed in tomato plants. Tomato fruits containing the TBI-HBS antigen were fed to experimental mice and, on days 14 and 28 post-feeding, high levels of HIV- and HBV-specific antibodies were present in the serum and feces of the test animals. Intraperitoneal injection of a DNA vaccine directing synthesis of the same TBI-HBsAg antigen boosted the antibody response to HIV in the blood serum; however, it had no effect on the high level of antibodies produced to HBV.

Keywords Hepatitis B virus · Human immunodeficiency virus · Oral plant-based vaccine · Transgenic tomato

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Introduction

Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) are the causative agents of serious diseases in humans. According to the WHO, HIV-induced acquired immunodeficiency syndrome (AIDS) kills 1–3 million people annually (UNAIDS 2002). The number of chronic HBV carriers is estimated to be 350 million people and the annual HBV morbidity rate exceeds 1 million (Michel 2002).

Vaccination is the most efficient method for protection against viral diseases and, as the mucosa is the first barrier to infection, stimulation of mucosal defenses is among the most important targets in disease prevention. Numerous studies have demonstrated that induction of systemic

immunity using the most widespread immunization by injection may efficiently prevent systemic infections, however, injection fails to protect mucosae. Mucosal immunization, on the other hand, most often stimulates both the mucosal and systemic immune responses (Chen 2000), and is accompanied by production of secretory IgA (sIgA) immunoglobulin, not synthesized during the systemic immune response. sIgA prevents the interaction of pathogens with surface receptors of mucosal cells. Mucosal immune responses of gastrointestinal, respiratory, and genital tracts are interconnected, therefore, if the immune response is stimulated in the intestine, the specific immune response to the antigen and production of the corresponding sIgA will be observed in the respiratory and genital tracts as well (Chen 2000).

Vaccines encapsulated within capsules of biodegradable material may be used for oral delivery and mucosal immunization. In addition to mechanical protection in the stomach, capsules are capable of releasing antigens gradually in the intestine. Plant-based oral vaccines are a new, promising direction in biotechnology (Daniell et al. 2001), and have several advantageous features over conventional injected vaccines (Streatfield and Howard 2003). The plant cell serves as a protective capsule for the antigen and, as there is no need for purification of recombinant proteins when the target genes are expressed in edible plant tissues, it is more economically sound to use transgenic plants for antigen production. Purification of recombinant antigens from other sources is a major factor contributing to cost. Human and animal pathogens are also absent in plants, contributing to safety. Finally, dried plant-based vaccines are easily stored and administered. The object of our study was to produce an oral, dried, plant-based vaccine using a combination of HBV and HIV antigens to concurrently protect against HBV and HIV.

HBsAg (hepatitis B surface antigen), produced in large quantities in liver cells of HBV-infected individuals and is capable of self-assembling into highly immunogenic isometric particles (Valenzuela et al. 1982). Plant-produced recombinant HBsAg accumulates intracelluarly as tubular structures in plant cells (Smith et al. 2003) and oral immunization with plant material expressing the HBsAg has been shown to be effective in eliciting a strong antibody response to HBV in mice (Kapusta et al. 1999; Richter et al. 2000; Kong et al. 2001) and humans (Kapusta et al. 2001).

An effective HIV vaccine requires both T cell epitopes, to stimulate an efficient $CD4^+$ T cell response and elicit cytotoxic lymphocytes, and B cell epitopes, to elicit protecting antibodies. Previously, we designed a novel synthetic HIV vaccine, *TBI*, expressed it in *Escherichia coli*, and evaluated its immunogenicity in mice (Eroshkin et al. 1993). *TBI* encodes an artificial polypeptide composed of immunogenic T- and B-cell epitopes of HIV-1 ENV and GAG proteins (T- and B-cell immunogens). ENV (gp41) is a transmembrane glycoprotein present in the envelope of HIV. GAG (p24) is a nuclear protein that is abundant in the viral particle. These proteins are a target for entry-blocking drugs and for stimulation of neutralizing antibodies that could contribute to protection against the virus. Antigenic determinants of cellular (amphipathic α-helices) and humoral immunity (flexible hydrophilic loop regions) of these two proteins were used to design the four-α-helix bundle of TBI (Eroshkin et al. 1993).

Here we report the production of transgenic tomato plants expressing a chimeric TBI-HBsAg antigen, which can potentially assemble into supramolecular structures composed of the HBsAg protein, and where the artificial polypeptide TBI may be located on the surface of the particles (Eroshkin et al. 1995; Nesterov et al. 2004). We previously detected, by electron microscopy, that TBI-HBsAg forms tubular structures in eukaryotic cells (Nesterov et al. 2004). Dried tomato tissue containing the antigen was evaluated as a candidate oral vaccine in experiments with laboratory animals. Preliminary research results were previously reported (Shchelkunov et al. 2004a, 2004b, 2005).

Materials and methods

DNA constructs

The artificial coding sequence *TBI-HBS* (encoding the TBI-HBsAg, Nesterov et al. 2004) was

amplified from the plasmid pTBIk2 (Eroshkin et al. 1995) using primers 5′-GATCGTC-GA***CCATGG***ATCTGCAGACTCATG-3′ and 5′-TCTAGA***AGATCT***GTTTAAATGTATAC-CCAAAGACAGAA-3′. These primers include sites for restriction endonucleases *Nco*I and *Bgl*II (bold italic). Polymerase chain reaction (PCR) was performed in a T-Gradient Thermocycler (Biometra GmbH, Düsseldorf, Germany), and the resulting amplicon was digested with *Nco*I and *Bgl*II restriction endonucleases, inserted into the plasmid pRT104 (Topfer et al. 1987), which was transformed into *E. coli* XL1-Blue cells. The DNA construct contained the strong constitutive transcriptional p35S promoter of *Cauliflower mosaic virus* (CaMV), the Kozak sequence (Kozak 1986) for efficient initiation of mRNA translation, the coding sequence of the TBI-HBS chimeric protein, and the mRNA polyadenylation signal of CaMV. It was excised from plasmid pRT*TBI-HBS*-6 (Fig. 1A, B) using the *Hin*dIII. This fragment was inserted into the binary vector pBINPLUS/ARS, a gift of Dr. Bill Belknap, USDA-ARS, Albany, CA, USA (Fig. 1C). The structure of the resulting construct, pBINp35S*TBI-HBS*#15, was confirmed by restriction analysis and nucleotide sequencing. *Agrobacterium tumefaciens* LBA4404 was transformed with the plasmid pBINp35S*TBI-HBS*#15 as described (Gelvin and Schilperoot 1998).

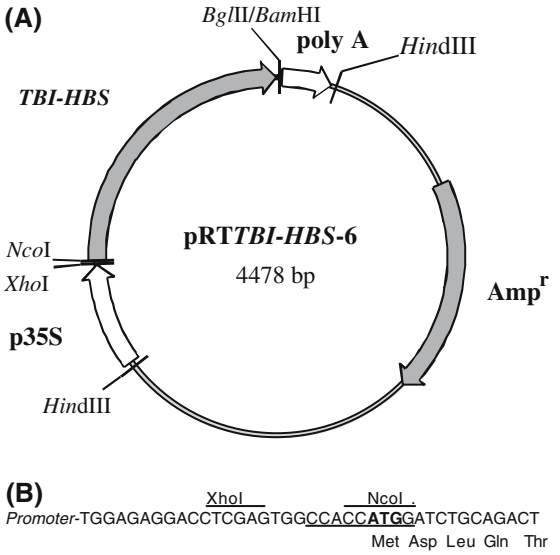

Fig. 1 Schematic diagram of the plasmid pRT*TBI-HBS*-6 carrying the *TBI-HBS* coding sequence: (**A**) p35S and polyA are sequences of the Cauliflower mosaic virus (CaMV) 35S transcriptional promoter and polyadenylation signal, respectively, and (**B**) nucleotide sequence at the region where p35S and *TBI-HBS* coding sequence are joined; the initiation triplet is bold-faced; Kozak sequence, underlined; *Xho*I and *Nco*I restriction endonucleases, overlined. (**C**) Schematic diagram illustrating the hybrid plasmid pBINp35S*TBI-HBS*#15: p35S and polyA, *CaMV* 35S RNA promoter and polyadenylation signal sequence, respectively; P-ubi3 and T-ubi3, promoter and terminator of *ubi3* gene, respectively; *ori* RP4 and *ori* ColE1, replication origins of the plasmids RP4 and ColE1; *NPTIII*, gene rendering bacterial cells resistant to kanamycin; and P-ubi3-*NPTII*-T-ubi3, hybrid gene rendering transformed plants resistant to kanamycin. (D) Schematic diagram of the TBI-HBS vaccine antigen. The location of T- and B-cell epitopes of the HIV-1 ENV and GAG proteins are shown as the shaded regions in the artificial TBI protein (Eroshkin et al. 1995). The T-cell epitopes (α-helical regions) are denoted by the open shading and the B-cell epitopes are denoted by boxed shading. The 226 aa HBsAg is fused to the 3′ terminus of the TBI protein as shown

Plant transformation

Explants of 15–20-day-old sterile seedlings of tomato cultivar *Lycopersicon esculentum* cv. Ventura were transformed using a modification of the flamingo-bill method (Pozueta-Romero et al. 2001). Briefly, explants were pricked with a needle containing a culture of *A. tumefaciens* LBA4404 (pBINp35S*TBI-HBS*#15) into the wound surface formed upon removal of the apical bud. Infected seedlings were cultivated *in vitro* for 15–20 days on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 10 mg thiamine/l, 0.05 mg kinetin/l, 0.2 mg gibberellic acid (GA_3)/l, 0.1 mg IBA/l, 3 mg Phytagel/l and supplemented with 50 mg kanamycin/l and 200 mg cefotaxime/l or media MSG containing MS salts, Gamborg's vitamins (Gamborg et al. 1976), 10 g sucrose/l, 0.5 g MES/l, 3 g phytagel/l and supplemented with 50 mg kanamycin/l and 200 mg cefotaxime/l. Regenerants that formed on the wound surface were excised and transferred to the MSG medium containing cefotaxime (100 mg/l), and placed at 24–26°C and illumination of 3500–4000 lux. Regenerants that survived and rooted on the selective medium were grown further, transferred to pots with soil, and planted either under hydroponic conditions

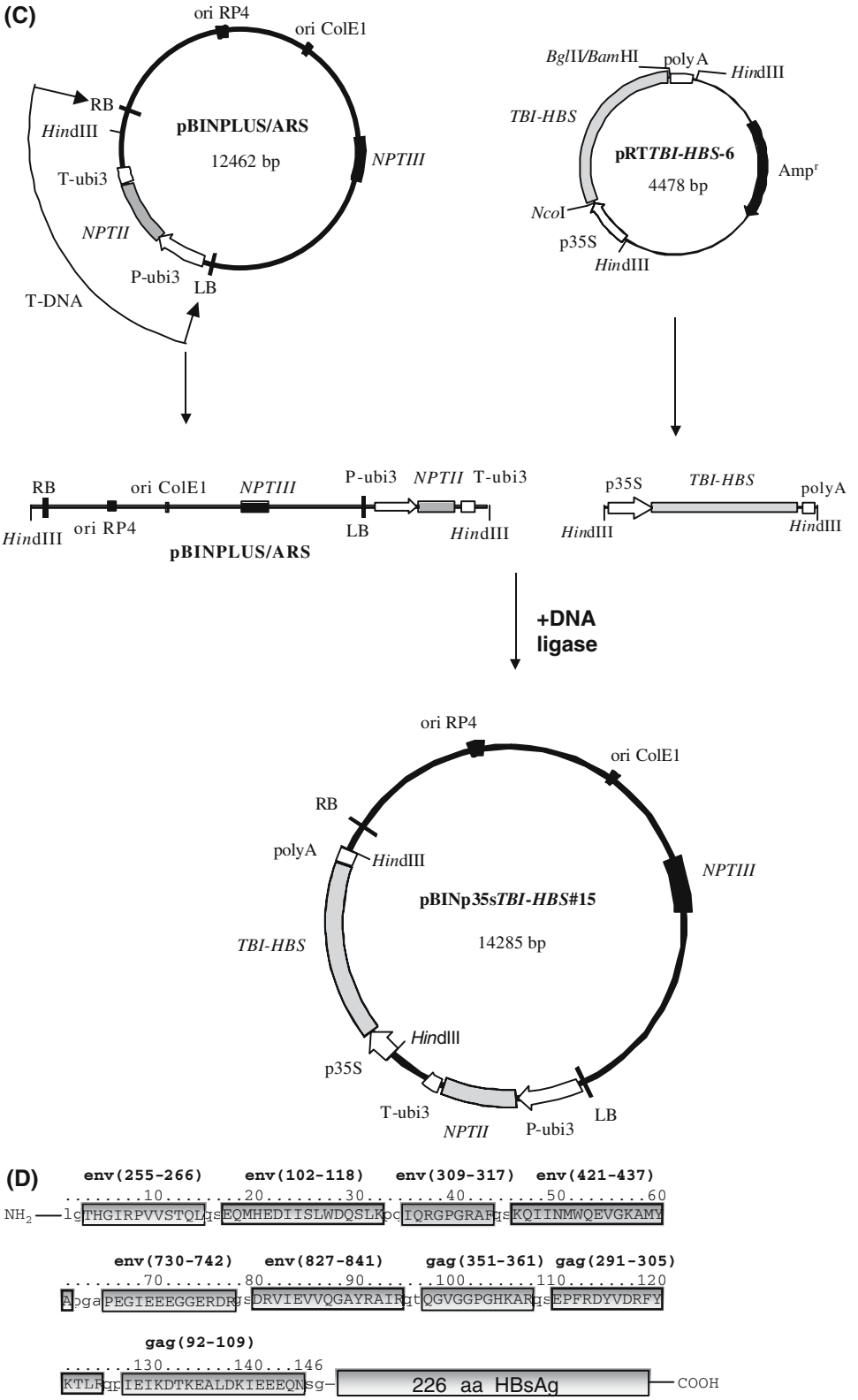

Fig. 1 Continued

in a phytotron or in an isolated greenhouse to produce fruits (T_0 generation).

Seeds of mature T_0 tomato fruits were germinated to produce 7-day-old seedlings on 0.5 MS medium supplemented with 100 mg/l cefotaxime. Roots of seedlings were removed, and the seedlings were transferred to the same medium containing 50 mg/l kanamycin. Seedlings that formed roots during 2–2.5 weeks on the kanamycin-containing medium were transferred for further growth and acclimation followed by replanting into an isolated greenhouse to produce fruits (T_1 generation).

Analysis of target gene integration into the plant genome

DNA was isolated from tomato leaves using CTAB (Lassner et al. 1989). Oligonucleotide primers 5′-CGACGTTGTCACTGAAGCG-3′ and 5′-AAGCACGAGGAAGCGGTCAG-3′ were used to confirm the presence of the *NPTII* gene in plant genomic DNA by PCR. The anticipated size of the amplicon was 487 bp. Primers 5′-ACGTAGATCGTTTCTATAAAACCCTGC GTGGG-3′ and 5′-AGGTTCCTTGAGCAGT AGTCGTGCAGGTTCT-3′ were used to detect the presence of *TBI-HBS* gene (anticipated amplicon size, 498 bp). Both PCR reactions comprised a hot start at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing for 1 min at 64°C, and elongation for 30 s at 72°C, using ReadyMix Taq PCR Reaction Mix with $MgCl_2$ (Sigma), Taq PCR Master Mix (Qiagen Gmbh, Düsseldorf, Germany), and kits obtained from Vector-Best (Koltsovo, Novosibirsk oblast, Russia).

Analysis of TBI-HBsAg production in tissues of transgenic plants

Protein extracts were obtained from tomato tissue according to Thanavala et al. (2005). Briefly, plant tissue (1 g) was ground with liquid N_2 and supplemented with 1 ml extraction buffer (0.05 M Na_2HPO_4 pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.3% Tween 20, and 4 mM PMSF). During thawing, the tissue was further ground with the pestle to homogeneous slurry and left overnight at 4°C for extraction. The extract was centrifuged for 30 min at 30,000 *g* at 4°C. The supernatant was transferred into new tubes, centrifuged for 40 min at 40,000 *g* at 4°C, and again transferred into new tubes. The tubes were placed on ice and samples were transferred onto strip plates of the test kits described below.

HBsAg was determined using a Recombahep B D0556 kit containing recombinant HBsAg and monoclonal antibodies (sensitivity 0.1 ng/ml; HBsAg standard was used; Vector-Best, Koltsovo, Novosibirsk oblast, Russia), designed for clinical diagnostics of hepatitis B, according to the manufacturer's instructions. Incubation with the conjugate was performed for 17 h at 4°C.

TBI was determined immunochemically with a Genscreen Plus HIV Ag/Ab (Bio-Rad, France) kit for HIV detection using manufacturers instructions. This kit is designed to detect antibodies to HIV-1 and HIV-2 and HIV-1 p24 (gag) antigen in human serum or plasma, as monoclonal antibodies to HIV-1 p24 and purified antigens are adsorbed in the wells.

Fruits of transgenic tomato line 13 (T_1 generation) and control non-transgenic tomatoes of cultivar *Ventura* were cut and dried in an OE-960/2 (Hungary) laboratory freeze-drier at a temperature of −50°C. Samples of dried tomato fruits were ground to homogeneity in a coffee mill for administration to laboratory animals.

Immunization of mice

Immunogenicity of the candidate oral vaccine was evaluated using three groups of BALB/c mice (50 animals each) weighing 15–20 g each, obtained from the Animal Breeding Facility at the State Research Center of Virology and Biotechnology Vector. Experimental mice received dried preparations of transgenic and non-transgenic tomato fruits, while the control group received the standard diet lacking tomatoes. All mice were fed a standard diet through the experiment. Prior to feeding the tomato preparations, mice were allowed to fast for 12 h. Immediately before feeding, the dried tomato fruit preparations were reconstituted with distilled water at 100 mg dried solids/ml and administered at a dose of 30 ng antigen (1 ml) into the esophagus via a catheter.

This procedure of feeding was chosen to standardize the antigen dose. Homogenates of dried tomato fruits were administered to animals on days 0, 14, and 28 of the experiment. On day 42, 10 animals of the group that received the preparation of transgenic plants were vaccinated with 50 μg of the plasmid pTBI-HBS, a candidate DNA vaccine (Pozdnyakov et al. 2003).

Total blood was collected on days 7, 14, 21, 28, 35, 42, 49, and 56 from five animals of each group, killed by decapitation with a guillotine. The serum samples were frozen and stored at −20°C. In addition, feces were collected during euthanasia, frozen, and stored at −20°C to assay for antibodies secreted by intestinal mucosa. On completion of the experiment, all the samples of peripheral blood and feces were assayed by ELISA for the presence of antibodies to HBsAg and HIV proteins using D-0562 and D-0172 kits (Vector-Best, Koltsovo, Novosibirsk oblast, Russia), respectively, following manufacturer's instructions. The HIV kit detects any antibodies to HIV-1 and HIV-2 using recombinant antigens and a recombinant conjugate. This kit does not contain calibration solutions with known concentration of antibodies to HIV antigens. The HBsAg kit detects any types of antibodies to the HBsAg using a standard recombinant antigen expressed in mammalian cell culture. This kit contains calibration solutions with known concentrations of antibodies to HBsAg. For the assay, fecal samples were defrosted at a temperature of 8°C, homogenized in physiological saline, and clarified by centrifugation at 10,000*g* for 15 min to determine the presence of antibodies in the supernatant by ELISA.

Results and discussion

Construction of the integration plasmid and plant transformation

The *TBI-HBS* hybrid gene (Nesterov et al. 2004) (Fig. 1D) was engineered into the binary vector pBINPLUS/ARS (Fig. 1C), under the control of the strong constitutive p35S promoter of CaMV. *A. tumefaciens* strain LBA4404 was transformed with the plasmid construct and the structure of the plasmid pBINp35S*TBI-HBS*#15. Of 2000 initial plant explants, only 26 regenerants rooted successfully on selective medium. They were transferred into pots with soil and planted either under hydroponic conditions in a phytotron or in an isolated greenhouse to produce fruits. T_0 seeds were germinated and planted on medium containing kanamycin. In total, seven T_1 plants were obtained and designated 11-1, 11-2, 13-1, 13-2, 13-3, 13-4, and 4. By PCR assay, the *NPTII* gene was reliably detected in plants 11-2, 13-1, 13-2, 13-4, and 4 (data not shown). The presence of the *TBI-HBS* and *NPTII* genes was verified by PCR analysis in lines 13-1, 13-2, 13-4, and 4 (data not shown).

Analysis of production of TBI-HBsAg antigen

Leaves and fruits of T_1 plants were shown to contain TBI-HBsAg antigen by ELISA (Fig 2A and B, respectively). The presence of TBI in these transgenic plants was demonstrated earlier by us (Shchelkunov et al. 2004a) when analyzing extracts of leaves and fruits using the EIA kit for HIV p24 protein. Clonal diversity in the level of target protein expression was observed (Fig. 2), which is a characteristic of transgenic plants (Hansen and Chilton 1999). In addition, variation was observed in detectable amounts of HbsAg in independent protein extractions from tissues of the same transgenic plant. Therefore, we isolated and assayed for the target protein in numerous replicates. Averaging of the results of the replicates allowed us to determine the mean value of HBsAg for each plant line (Fig. 2). The content of TBI-HBsAg chimeric protein in the dried preparations was determined by ELISA using Recombahep B D-0556 kit (Vector-Best, Koltsovo, Novosibirsk oblast, Russia); tomato fruits from line 13 accumulated no less than 0.3 ng antigen per 1 mg powder.

Analysis of specific immunogenicity of transgenic tomato fruits

Immunogenicity of the candidate vaccine was evaluated using three groups of BALB/c mice as described in Materials and methods. Antibody levels against HBsAg and HIV in animal blood

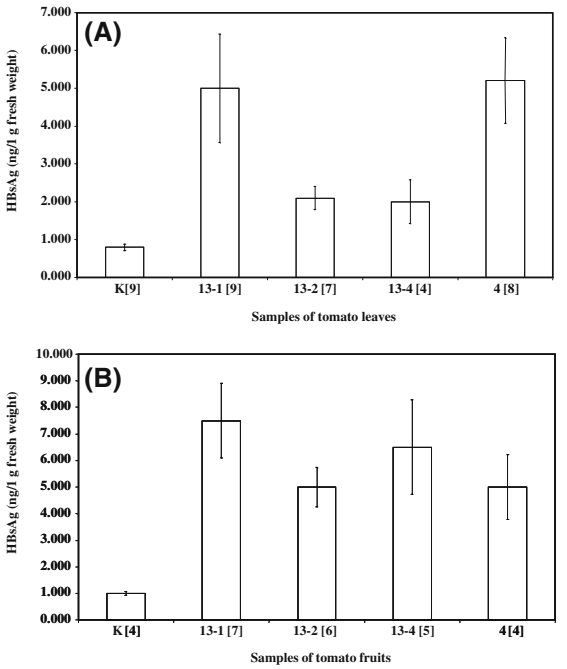

Fig. 2 Determination of HBsAg in (**A**) leaves and (**B**) fruits of tomatoes transformed with *TBI-HBS* gene (T_1 generation): K, non-transgenic plant; 13-1, 13-2, 13-4, and 4, transgenic plants. Numbers of replicates of extracts and analyses for each plant are shown in square brackets. Mean values are shown and standard errors of measurement by EIA are indicated by bars

sera and feces were determined up to day 56 of the experiment. Levels of antibodies to HBsAg in mouse blood serum increased after the second feeding and remained high until the end of experiment (Fig 3A). The mucosal immune response (secreted antibodies) to HBV, detected in feces, started earlier, after the first feeding, and remained at a high level until the end of experiment (Fig. 3B).

The same sample of tomato fruit induced the humoral immune response to HIV after the second feeding (Fig. 4A). Although the titer determined using the enzyme immunoassay (EIA) system for detection of antibodies to HIV was, in this case, lower compared with the level of antibodies to HBsAg, they significantly exceeded the control values (Fig. 4A). When analyzing feces, induction of the secreted antibodies (mucosal immune response) began considerably earlier as compared to the antibodies detected in the blood serum (Fig. 4B), and similar to what was observed with HBsAg.

To determine whether combined use of oral plant-derived and injected DNA vaccines would boost the immune response to TBI-HBsAg, 50 μg of the plasmid pTBI-HBS, expressing TBI-HBsAg polypeptide in mammalian cells (Pozdnyakov et al. 2003), was administered intraperitoneally on day 42 to 10 animals from the group that received the preparation of transgenic tomato fruits. Additional administration of pTBI-HBS had no effect on the high antibody response to HBsAg that was observed upon feeding with transgenic tomatoes (see Fig. 3A, B), whereas the humoral immune response against HIV increased considerably (Fig. 4A). As was anticipated, injection of the plasmid pTBI-HBS did not influence production of the antibodies to HIV secreted by intestinal mucosae (Fig. 4B).

Oral immunization with plant-based HIV vaccines gave mixed results. For example, McLain et al. (1996) reported the induction of low levels of HIV-specific neutralizing antibodies in mice vaccinated subcutaneously with a plant virus particle expressing a 22 amino acid epitope of HIV glycoprotein gp41 fused to the plant virus capsid protein; injection in the presence of adjuvant increased the levels of antibody produced (McInerney et al. 1999). Intranasal inoculation of this same antigen in the presence of an adjuvant resulted in HIV-1-specific antibodies in the serum and feces, whereas oral immunization of the chimeric virus was less effective at stimulating serum antibodies and no fecal antibodies were found (Durrani et al. 1998). Karasev et al. (2005) prepared an oral vaccine by transient expression of the HIV Tat protein in spinach using a plant viral-based vector. None of the mice fed on spinach leaves expressing the Tat protein developed measurable antibodies. However, following DNA vaccination with a Tat protein-expressing plasmid, mice that previously received oral Tat developed higher serum antibody levels than those not fed the plant-based vaccine. Our results with administration of pTBI-HBS showed no effect on the intestinal mucosal antibody response to HIV, whereas the humoral immune response against HIV increased considerably (Fig 4A). Therefore, the difference in the nature of the HIV-1 antigens used can have a significant effect on immunogenic responses in test animals.

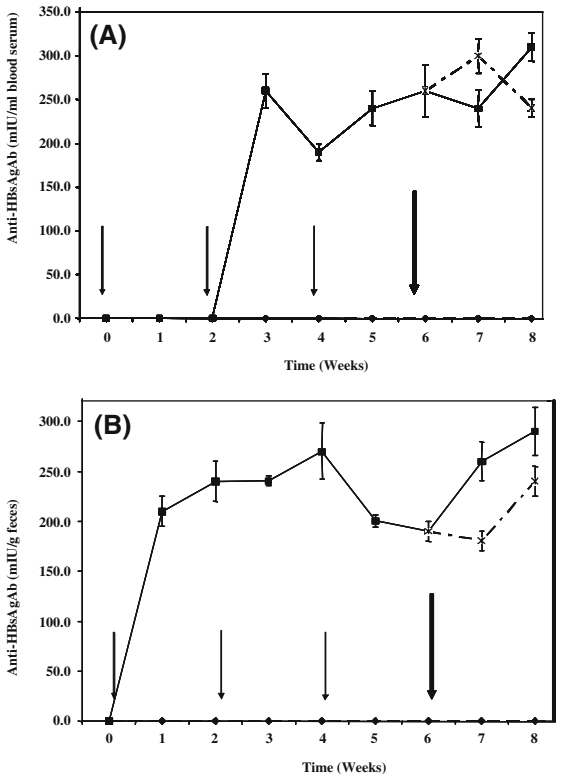

Fig. 3 Dynamics of anti-HBsAg antibody levels in (**A**) blood serum and (**B**) feces of mice upon feeding with transgenic and non-transgenic tomatoes and injection of DNA vaccine: fine vertical arrows indicate days when tomatoes were fed to animals; bold short arrow, immunization with DNA vaccine. Mean values are shown and standard errors of measurement by EIA are indicated by bars. (♦) non-transgenic tomato; (■) transgenic tomato; (×) DNA vaccine

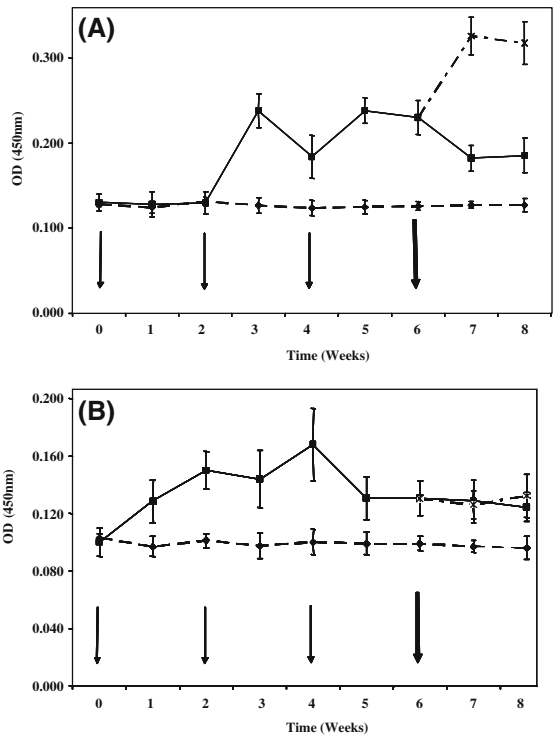

Fig. 4 Dynamics of anti-HIV antibody levels in (**A**) blood serum and (**B**) feces of mice upon feeding with transgenic and non-transgenic tomatoes and injection of DNA vaccine: fine vertical arrows indicate days when tomatoes were fed to animals; bold short arrow, immunization with DNA vaccine. Mean values are shown and standard errors of measurement by EIA are indicated by bars. (♦) non-transgenic tomato; (■) transgenic tomato; (×) DNA vaccine

Our studies demonstrate that oral administration of dried tomato tissue containing a chimeric HIV/HBV vaccine antigen stimulates both serum and secretory HIV- and HBV-specific antibodies in mice, indicating activation of both blood-borne and mucosal immune responses (Figs. 3 and 4). In addition, our results demonstrate, for the first time, that a candidate oral vaccine against the two viruses concurrently can elicit immune responses in test animals. Additional administration of a DNA vaccine is reasonable only in the case of a low immune response to the target antigen of HIV (Fig. 4A) and has no detectable effect on the high level of immune response to HBsAg (Fig. 3A, B). This plant-produced protein has the potential for development into a candidate vaccine for vaccination of humans concurrently against both HBV and HIV.

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